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RESEARCH ARTICLE OPEN ACCESS

Long-Term Drought Persistently Shifts Plant and Soil Microbial Communities but Has Limited Impact on CO₂ Fluxes Under Subsequent Drought

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ABSTRACT

Droughts are increasing with climate change, affecting the functioning of terrestrial ecosystems and limiting their capacity to mitigate rising atmospheric CO₂ levels. However, there is still large uncertainty on the long-term impacts of drought on ecosystem carbon (C) cycling, and how this determines the effect of subsequent droughts. Here, we aimed to quantify how drought legacy affects the response of a heathland ecosystem to a subsequent drought for two life stages of *Calluna vulgaris* resulting from different mowing regimes. We imposed a subsequent drought in a long-term (20 years) drought field experiment combined with different mowing years. We hypothesised that drought legacy would reduce the impact of a subsequent drought on ecosystem respiration (ER) through shifts in microbial community composition, and we expected a stronger effect of drought legacy on building stage *Calluna* (mowed in 2013) than on seedlings (mowed in 2020), with knock-on effects for net ecosystem exchange (NEE) and ER. We found that drought legacy persistently shifted soil bacterial and fungal communities, but the subsequent drought had minimal effect. Drought legacy also shifted plant community composition, with the strongest effect of subsequent drought on the building stage of *Calluna*. Subsequent drought reduced all CO₂ fluxes independent of drought legacy, and this effect was most pronounced in the building stage of *Calluna*. The observed strong and persistent shifts in soil microbial communities as a result of 20 years of summer drought did not explain ecosystem CO₂ fluxes, which were determined by changes in plant communities. Thus, our findings show a mismatch between aboveground and belowground responses to drought, and highlight that older heathlands are more vulnerable to drought, reducing their CO₂ uptake capacity in the crucial phase of ecosystem C stock accumulation. These findings give insight into the consequences of long-term drought for ecosystem C cycling and its response to future drought.

1 | Introduction

Drought events, which are becoming more common with climate change, can have strong effects on the composition of plant and soil microbial communities, impacting important ecosystem processes that can feed back to climate change through affecting the uptake and loss of CO₂. Droughts have been predicted to

be the climate extreme events with the strongest and most widespread effects on terrestrial carbon cycling (Frank et al. 2015). Still, despite many studies summarizing mostly negative effects of drought on ecosystems, there is high uncertainty on how drought impacts land-based CO₂ feedbacks (Sippel et al. 2018), and how these can be incorporated into global C cycling models (Anderegg et al. 2020). There is particular uncertainty on

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the longer term and indirect effects of drought on ecosystem C uptake (Anderegg et al. 2020), while most studies to date have focused solely on understanding short-term effects of drought, and sometimes its subsequent recovery. The IPCC (2023) predicts that drought periods are becoming longer, more intense and more frequent (Samaniego et al. 2018). Thus, it is crucial to improve our understanding of the long-term effects of prolonged drought periods and how they affect ecosystem responses to a subsequent drought, and the implications for ecosystem C cycling.

Drought can affect ecosystem C uptake, or plant photosynthesis and the subsequent incorporation into plant biomass or allocation to soils and soil organisms, via multiple mechanisms that operate on different timescales. Drought reduces plant photosynthesis and growth; thus, reducing ecosystem C uptake, and because plant species differ in their susceptibility to drought, these impacts can ultimately result in shifts in plant community composition. The ability to quickly close their stomata is the first defence of plants against drought. This happens faster in species with high stomatal density and photosynthesis rates and thus fast growth rates. However, this ability trades off with a higher sensitivity to low leaf water potential and thus leaf damage and xylem embolism (Henry et al. 2018). Stomatal closure reduces water loss through transpiration but also stops CO₂ uptake and thus photosynthesis (Henry et al. 2018). Light capture then exceeds CO₂ uptake rates, resulting in oxidative damage through the build-up of reactive oxygen species (ROS). These changes can leave a 'memory' in plants that primes these response mechanisms and may reduce the impact of a subsequent drought through activation of key hormones and changes in gene expression (Jacques et al. 2021; Walter et al. 2013). Other strategies that may reduce the impact of subsequent drought in individual plants are increased root-to-shoot ratio, deeper rooting, or increased tillering (Müller and Bahn 2022; Walter et al. 2013). Conversely, accumulated drought damage as well as increased vulnerability to pests and pathogens may increase the impact of subsequent drought (Müller and Bahn 2022).

Plant drought resistance traits are correlated with plant growth strategies, with slow-growing, well-defended plants generally being able to maintain water potential through deeper rooting and tolerate lower water potential before embolism, resulting in the ability to maintain photosynthesis (Henry et al. 2019; Volaire 2018). These differences in drought tolerance ultimately lead to shifts towards a higher abundance of species with these traits under drought (Mackie et al. 2019; Malik and Bouskill 2022; Ouédraogo et al. 2013). This may decrease the impact of a subsequent drought, but also reduce overall photosynthesis rates and ecosystem C uptake (Frank et al. 2015). In systems with long-lived vegetation, such as forests, a trade-off has been found for the ability to withstand one drought vs. the ability to withstand multiple droughts, with angiosperms having a stronger growth reduction after one drought, but smaller growth reductions after multiple droughts, while gymnosperms showed increased sensitivity with recurring droughts (Anderegg et al. 2020). Moreover, primary forests, which generally consist of older trees in less disturbed sites, have been shown to be less impacted by drought compared to secondary forests, which consist of younger trees (Wolf et al. 2023). Similarly, younger trees have

been found to be more reduced in their growth under drought than older trees, potentially because of their shallower, smaller root systems (Au et al. 2022). In contrast, ageing plants will be less efficient in their ROS scavenging mechanisms, thus likely building up more drought-induced tissue damage (Pérez-Llorca and Munné-Bosch 2021; Rankenberget al. 2021). However, we still do not know how plants and ecosystems of different ages respond to repeated drought.

Drought also affects soils and their bacterial and fungal communities, and these changes can alter ecosystem C outputs through affecting processes of decomposition, nutrient cycling and microbial respiration. Drought itself reduces microbial activity and respiration, and generally promotes the abundance of fungi relative to that of bacteria, causing more prominent shifts in bacterial communities than in fungal communities (Barnard et al. 2013; de Vries et al. 2013, 2018; Knight et al. 2024; Naylor and Coleman-Derr 2018). When a drought ends, bacterial activity and community composition tend to recover quickly than those of fungi (Barnard et al. 2013; Cordero et al. 2023; Knight et al. 2024), but recurring droughts of increasing duration or intensity seem to shift both fungal and bacterial communities to a state that does not return to control communities (Canarini et al. 2024; Cordero et al. 2023).

Persistent shifts in soil fungal and bacterial communities under long-term or repeated drought can also be driven by drought-induced changes in plant growth and community composition, and the resulting alterations in belowground C inputs. Drought reduces plant photosynthesis and the amount of C that is allocated belowground (Hasibeder et al. 2015), which has been further linked to a reduction in C transfer to soil bacterial but not to fungal communities (Fuchslueger et al. 2014). However, while ongoing drought affects both microbial respiration and plant C inputs, persistent shifts in fungal and bacterial communities can continue to affect ecosystem functioning after drought has ceased. Short-term drought has been shown to shift microbial communities towards fungal dominance, resulting in increased resistance to subsequent drought (de Vries et al. 2012). Chronic drought exposure persistently changes microbial physiology and functioning (Canarini et al. 2021), with drought adaptation resulting in faster bacterial growth rates after rewetting (Cordero et al. 2023; de Nijs et al. 2019), while total respiration quickly recovers to control levels. But in addition to these direct effects of changes in microbial communities on C cycling processes, shifts in fungal and bacterial communities can also feedback to plant growth (de Vries et al. 2023; Kaisermann et al. 2017), thus indirectly affecting ecosystem C uptake, as well as its response to subsequent drought (de Vries et al. 2012).

In heathlands, ecosystems that are amongst the oldest cultural landscapes of Europe, long-term drought has been shown to have lasting effects on both above and belowground ecosystem processes that can influence C cycling (Gliesch et al. 2024). Heathlands are dominated by the ericoid shrub *Calluna vulgaris* (hereafter *Calluna*), but are increasingly threatened by nitrogen (N) deposition and the accompanying encroachment of grasses, land conversion and climate change (Fagúndez 2013). Historically, heathlands have evolved through thousands of years of practices of forest clearing, grazing, turf cutting, burning and harvesting vegetation for fodder or fuel (Webb 1998).

Currently, mowing is a management practice used to promote the regeneration of *Calluna* and prevent grass encroachment (Schellenberg and Bergmeier 2021). Mowing creates patches of *Calluna* at different life stages that can directly influence ecosystem C stocks, with peak values found for young and middle-aged *Calluna* (Kopittke et al. 2013). Moreover, studying C fluxes in a similar chronosequence of *Calluna* life stages, Li, Larsen, et al. (2023) have shown that C uptake from *Calluna* peaks at around 12 years, which coincides with the building stage of its life cycle (10–15 years), and it then declines, turning the ecosystem into a C source at around 19 years. However, these early *Calluna* life stages are also the ones most vulnerable to drought in comparison to older *Calluna* (Meyer-Grünefeldt et al. 2015), suggesting that drought could significantly reduce heathland C uptake potential, depending on *Calluna* life stage. Importantly, previous work found that chronic summer drought reduced soil C under old (> 20 years) *Calluna* plants, but not under intermediately aged and young plants (Gliesch et al. 2024). Moreover, both fungal and bacterial communities were affected by drought as well as by the age of *Calluna* plants (Gliesch et al. 2024). However, we do not know whether these changes affect the ecosystem's response to subsequent drought, and what the relative role is of drought legacies on plant growth and soil microbial communities.

Here, we sought to understand how drought legacy affects ecosystem response to subsequent drought, and whether this depends on the age of the vegetation. We did this using a unique long-term drought field experiment in a heathland ecosystem, in which large plots (4 × 5 m) have been exposed to summer drought versus control conditions for more than 20 years. A long-term experiment like this provides crucial understanding into the ecological mechanisms underpinning ecosystem response to drought (Blanc and Thrall 2024). We quantified how the legacy of 20 years of summer drought determined the response of CO₂ fluxes, plant communities and soil microbial communities to a subsequent drought. We compared plots that were mowed in 2020 or in 2013, and thus represented young (seedling stage) and older (building stage) *Calluna* vegetation. We hypothesised that drought legacy decreases the impact of a subsequent drought on ecosystem respiration because of a shift towards drought-adapted soil microbial communities caused by chronic drought. We expected to see a stronger effect of drought legacy on older than on young *Calluna*, with overall reductions of net ecosystem exchange (NEE) and ecosystem respiration (ER) in response to both legacy and subsequent drought at the older (building stage) *Calluna*. Also, we hypothesised that young vegetation would be more susceptible to subsequent drought, thus reducing its C uptake more than older vegetation. Thus, we expected plant community responses to determine drought impacts on photosynthesis and NEE, while shifts in soil bacterial and fungal communities would determine drought impacts on ecosystem respiration.

2 | Materials and Methods

2.1 | Experimental Site

We used a long-term drought experiment that was originally set up in 1998 in a managed heathland in Oldebroek, the Netherlands (52°24'N, 05°55'E). The 1998 experimental design

included three original control and three original drought plots of each 20 m² (Beier et al. 2004). From 1999 to 2018, a retractable roof cover imposed a drought treatment for ca. 3 months during the growing season in the drought plots, which reduced average annual precipitation by ca. 33% and soil moisture by ca. 60% (de Nijs et al. 2019). These plots have been subdivided and mowed at different time points, thus creating distinct plant communities dominated by *Calluna* at different growth stages: young (mowed in 2013), intermediate (mowed in 2009) and old (nonmowed at least since 1984). In August 2020, we mowed half of the originally nonmowed part of the plot to create a new 'zero' plot for the growth of *Calluna* seedlings and other plant species. On the same day, we installed a permanent roof to exclude 50% precipitation in all original plots. The acrylic sheet roof (which let > 90% UV light through) was installed in the middle of each of the original control and drought plots, covering half of all different mowing treatments (1998, 2009, 2013 and 2020). Consequently, each original plot was now subdivided into droughted and non-droughted parts, which allowed us to compare how drought legacy influences the response to a subsequent drought treatment for different mowing years (see Figure 1 for experimental design and Figure S1 for a picture of one of the plots). In each experimental unit, we installed TMS-4 loggers (Wild et al. 2019) to monitor volumetric soil moisture, soil temperature at ground level and air temperature 15 cm above ground during the 3 years of the subsequent drought experiment. For the purposes of this manuscript, we have focused on the parts of the plots mowed in 2020 and in 2013, which represent young (seedling) and older (building) stages of *Calluna* (highlighted in red in Figure 1).

2.2 | Vegetation Surveys, Soil Collection and Soil Nutrients and Carbon Pools Measurements

Every year (from 2021 to 2023), at peak biomass (July), we recorded vascular plant species cover and bryophyte cover (hereafter referred to moss) in a 0.25 m² area inside a metal frame used for CO₂ flux measurements in the 2020 and 2013 mowed parts of the plots (total of 24 observations for vegetation, 2 legacy treatments * 2 subsequent drought treatment * 2 mowing years * 3 replicates). For the three consecutive years, at the same time that plant species cover was recorded, individual soil cores of 1.5 cm from 15 cm depth were taken from the 2013 and 2020 mowed parts of the plots and pooled for nutrient and C pool analysis (ca 9 cores per plot). Soil samples were sieved at 2 mm and kept at 4°C before being analysed. From these sieved soil samples, we separated 1.5 mg for DNA analysis and kept it at –20°C until DNA extractions could be conducted. To measure plant-available N and P, we used a 1 M KCl extraction with a 1:5 fresh soil: solution ratio and measured the extracts in an auto-analyser (San ++, Skalar, Breda, The Netherlands); pH was measured with a pH meter (Consort C831) in unfiltered water extracts done with a 1:1.5 fresh soil: MiliQ water ratio. After filtration of water extracts through a 0.45 µm filter, we measured dissolved organic N (DON) in the auto-analyser and dissolved organic C (DOC) in a Vario TOC cube analyser (Elemental GmbH, Langenselbold, Germany). Soil microbial N and C were measured in 0.05 M K₂SO₄ extracts following a 24 h chloroform fumigation and corrected using a kec factor of 0.54 and 0.45 respectively (Brookes et al. 1985; Vance et al. 1987).

Experimental design

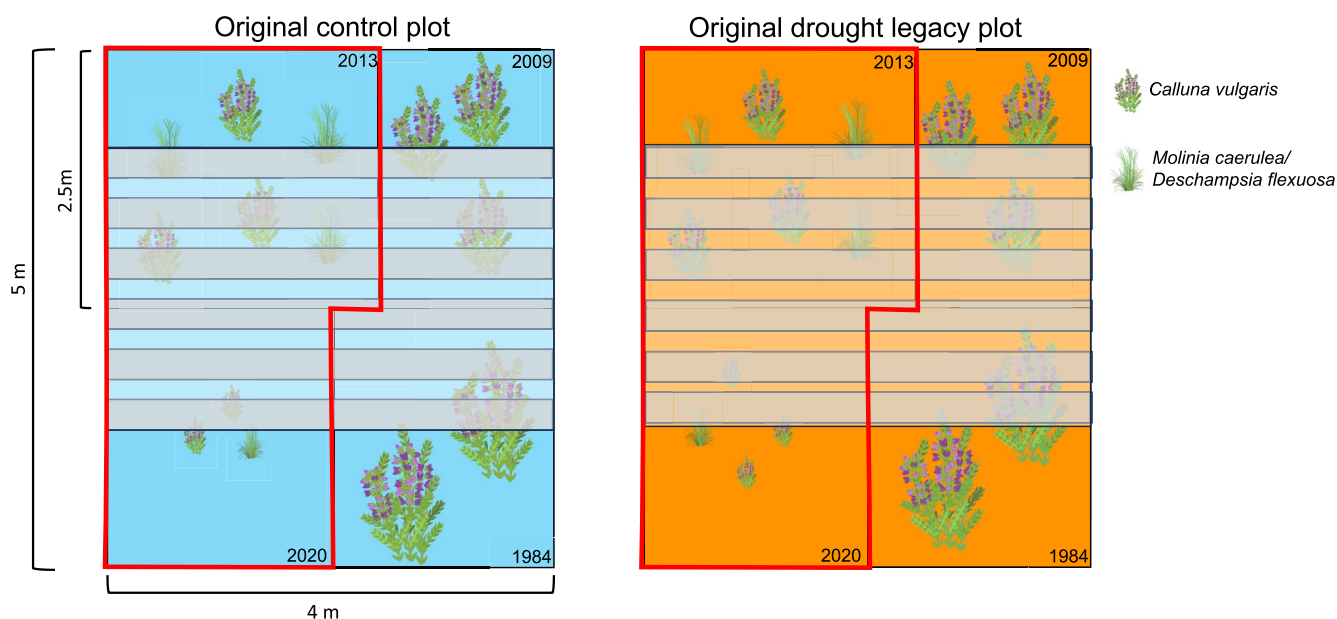


FIGURE 1 | Experimental design of the long-term drought experimental plots in Oldebroek, the Netherlands. The experimental plots have been mowed at different time points: 2009, 2013 and 2020, and there is a part of the plots that has not been mowed since at least 1984, leading to different growth stages of the vegetation. In August 2020, sheets of an acrylic roof were placed on top of every plot, covering approximately 50% of the entire plot area and allowing for all possible comparisons of original drought treatment and subsequent drought treatment at different mowing times. For this study, we focused on the marked red areas of the figure, which represent areas mowed in 2013 and 2020 that were original control plots (blue) or had a history of 18 years of drought (orange) and that were subdivided into droughted and nondroughted parts with the addition of the new acrylic roofs in August 2020. These allowed us to compare how drought legacy influences the response to a subsequent drought treatment for two different mowing years (2013 and 2020).

2.3 | Carbon Fluxes Measurements

To detect ecosystem changes in CO_2 fluxes, 24 permanent stainless-steel bases with dimensions of $0.5 \times 0.5 \text{ m}$ were embedded approximately 12 cm into the ground in August 2020 in the 2013 and 2020 parts of the plots, underneath the new roof and outside of it, enclosing soil and vegetation. During measurements, a 150 L transparent acrylic chamber ($0.5 \times 0.5 \times 0.6 \text{ m}$) was placed on the steel base, similar in size to the ones used in Li, Larsen, et al. 2023 and Li, Tietema, et al. 2023. Two air plugs were inserted into the chamber, one for air outlet and the other for air inlet, and a circulation fan was fitted in the chamber to provide proper air mixing (Christiansen et al. 2011). The chamber was connected to an EGM-5 Portable CO_2 Gas Analyzer Monitor (PP Systems, Amesbury, MA, USA) in a closed circuit. For 180 s, the chamber was placed on the steel base, and the inside CO_2 concentration was recorded at 1-s intervals. Simultaneously, the average of photosynthetically active radiation (PAR) during the 180 s was recorded using a LI-250 Light Meter and LI-190SA Quantum Sensor (LI-COR Biosciences, Lincoln, USA). A complete measurement cycle consisted of one 180 s cycle in light and one in darkness. During the light period, net ecosystem exchange (NEE) was measured, and with a dark chamber (same dimensions as the light one but not allowing any light through), we measured ecosystem respiration (ER). Measurements using the dark chamber were performed immediately after the light one for the same amount of time (180 s). The difference between these two fluxes (NEE- ER) is the CO_2 flux from photosynthesis.

All CO_2 fluxes were measured over three consecutive years (2021–2023) in the 2013 and 2020 mowed parts of the plots during the growing season. Generally, there was a 30-day interval between measurements in a growing season, which was between the months of May and September for the years 2021 and 2023, and between March and October for 2022. A total of 17 measurement days were done during the 3-year experiment. All plot measurements were conducted on the same day in a random block-wise cycle between 10:30 a.m. and 2:30 p.m. We used the *flux* package in R (Jurasinski et al. 2022) to calculate CO_2 fluxes with the function *fluxx* for nonsteady-state chambers with the *pd* parameter set to 0.8 (only 80% of measurements included). This function identifies the most linear part of the concentration development while excluding the first high-frequency fluctuations of the measurements and chooses the model with the highest r^2 (Jurasinski et al. 2022). For all respiration and NEE CO_2 flux measurements, we have set a minimum inclusion parameter for each model of $r^2 > 0.6$.

2.4 | Soil DNA Extraction, Amplification and Sequencing

We extracted microbial DNA from approximately 0.25 g of subsampled soil using the DNeasy PowerSoil Kit (QIAGEN GmbH., Hilden, Germany), following the manufacturer's instructions and investigated microbial community composition with amplicon sequencing. The bacterial 16S ribosomal DNA

region was amplified with the primer pair 515F–806R, and the fungal internal transcribed spacer 1 region (ITS1) was amplified using the primer pair ITS1-F–ITS2. The PCR reactions were conducted with a mixture containing 13.6 μ L of MQ water, 2 μ L of DreamTaq Buffer (10X), 2 μ L of 1 mM dNTPs, 0.2 μ L of DreamTaq DNA Polymerase (5 U/ μ L) (Thermo Fisher Scientific), 0.6 μ L of each primer (10 μ M for both forward and reverse primers) and 1 μ L of extracted DNA normalized to 5 ng/ μ L. The thermal cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 25 cycles for 16S and 30 cycles for ITS1 of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min. The amplification products were verified by electrophoresis, purified using the AMPure XP Kit (with a sample to XP ratio of 1:0.6, Agencourt), and their concentrations were measured with a Qubit fluorometer (Thermo Fisher Scientific Inc., Waltham, USA). The purified PCR products were sent to the Genome Research Core of the University of Illinois for a second PCR process and library construction. 16S samples were sequenced for 150 bp using an Illumina MiniSeq platform and ITS samples were sequenced for 250 bp using an Illumina MiSeq platform.

2.5 | Bioinformatics

Pair-end sequences for both sets of samples from 16S and ITS were processed with the *dasnake* pipeline (Weißbecker et al. 2020) using ‘cutadapt’ command for primer removals and DADA2 (Callahan et al. 2016) for quality filtering, pair-end merging and chimera removals. For 16S sequences, the min overlap parameter for pair merging was set at 4, while for ITS it was set at 20. Taxonomic assignment within the *dasnake* pipeline was performed by *mothur* for both amplicon sets; for 16S, the database used was SILVA (Quast et al. 2013), while for ITS it was UNITE (Kõljalg et al. 2019; Nilsson et al. 2019). The 16S dataset was filtered to exclude singletons, doubletons and reads that occurred in less than 12 samples, as well as samples that had less than 200 reads per sample recorded, which gave a final number of 72 samples with a total of 10,495 amplicon sequence variants (ASVs). The ITS dataset was filtered to exclude singletons and doubletons, and reads that occurred in less than 11 samples, which gave a final number of 1885 ASVs and 72 samples.

2.6 | Statistical Analysis

All statistical analyses were performed in R version 4.4.1 (R Core Team 2024). We used linear mixed-effect models to test for the fixed effects of drought legacy, subsequent drought and mowing year on the different CO₂ fluxes (ER, NEE and Photosynthesis) using a nested random term of plot/month/year with the function ‘lme’ using the maximum likelihood (ML) approach of the *nlme* package (Pinheiro et al. 2022) followed by type III ANOVA with the function ‘Anova’ from the *car* package (Fox and Weisberg 2019). Post hoc tests of significant treatment effects were performed by the function ‘emmeans’ of the package *emmeans* (Lenth 2022). For NEE and Photosynthesis, we included the average PAR value for each measurement as a co-variable and used square root to transform the response variables of CO₂

fluxes to improve model assumptions, which were checked visually for normal distribution of residuals. The effects of drought legacy, subsequent drought, mowing year and year of sampling and their interactions, on plant community composition were analysed with a PERMANOVA based on Bray–Curtis distances of log-transformed plant cover data and using plot as a block effect with the function ‘adonis2’ in the package *vegan* (Oksanen et al. 2020). The same function and model structure were used to analyse the effects of the same variables on soil microbial community composition, with a Euclidean distance matrix for clr-transformed 16S data for soil bacterial communities and a Bray–Curtis distance matrix for ITS data for soil fungal communities. To visualize differences in soil microbial community composition, we performed a PCA with the clr-transformed data for 16S bacterial communities and an NMDS for ITS fungal communities.

2.7 | Structural Equation Models for Drivers of CO₂ Fluxes

To test for the mechanisms governing NEE and ER responses to drought legacy and subsequent drought, we constructed two separate structural equation models (SEMs), one for each response variable, based on prior knowledge from the literature and using linear-mixed effect models with year and plot as nested random effects. The a priori SEMs with the main fixed effects are illustrated in Figure S2. For all models, the final response variable of NEE or ER is an average for the growing season, as we only had one measurement of plant and soil microbial community composition to relate these values to. Briefly, in our models, drought legacy and subsequent drought both directly affect soil bacteria and fungi community composition (represented by the scores of the first axis of the ordination based on all samples from the three sampled years in Figures 2 and 3, that is, PC1 for bacteria and NMDS 1 for fungi), which then are directly related to changes in ecosystem CO₂ fluxes (both NEE and ER). Drought legacy and subsequent drought also indirectly influence CO₂ fluxes via their effects on the plant community composition (represented by scores of the first axis of the PCA based on species cover data for the three sampled years in Figure 4), which can also influence both soil fungi and soil bacteria communities. Finally, plant cover can also affect the microclimate of the plot, here represented by changes in temperature, which can then indirectly influence NEE and ER through changes to soil fungi and bacteria community composition. Temperature in the SEM is represented by the average temperature at soil level (0 cm) across the growing season. SEMs were fit using the piecewise SEM package (Lefcheck 2016), where AIC values as well as d-sep tests were used to optimize model selection, and model fits were evaluated with Fischer’s C Score, where $p > 0.05$ represents a significant fit of the model.

3 | Results

3.1 | Experimental Conditions

The subsequent drought treatment reduced soil moisture on average by ca. 20% in droughted plots compared to control plots during the growing season (Figure S3). However, the

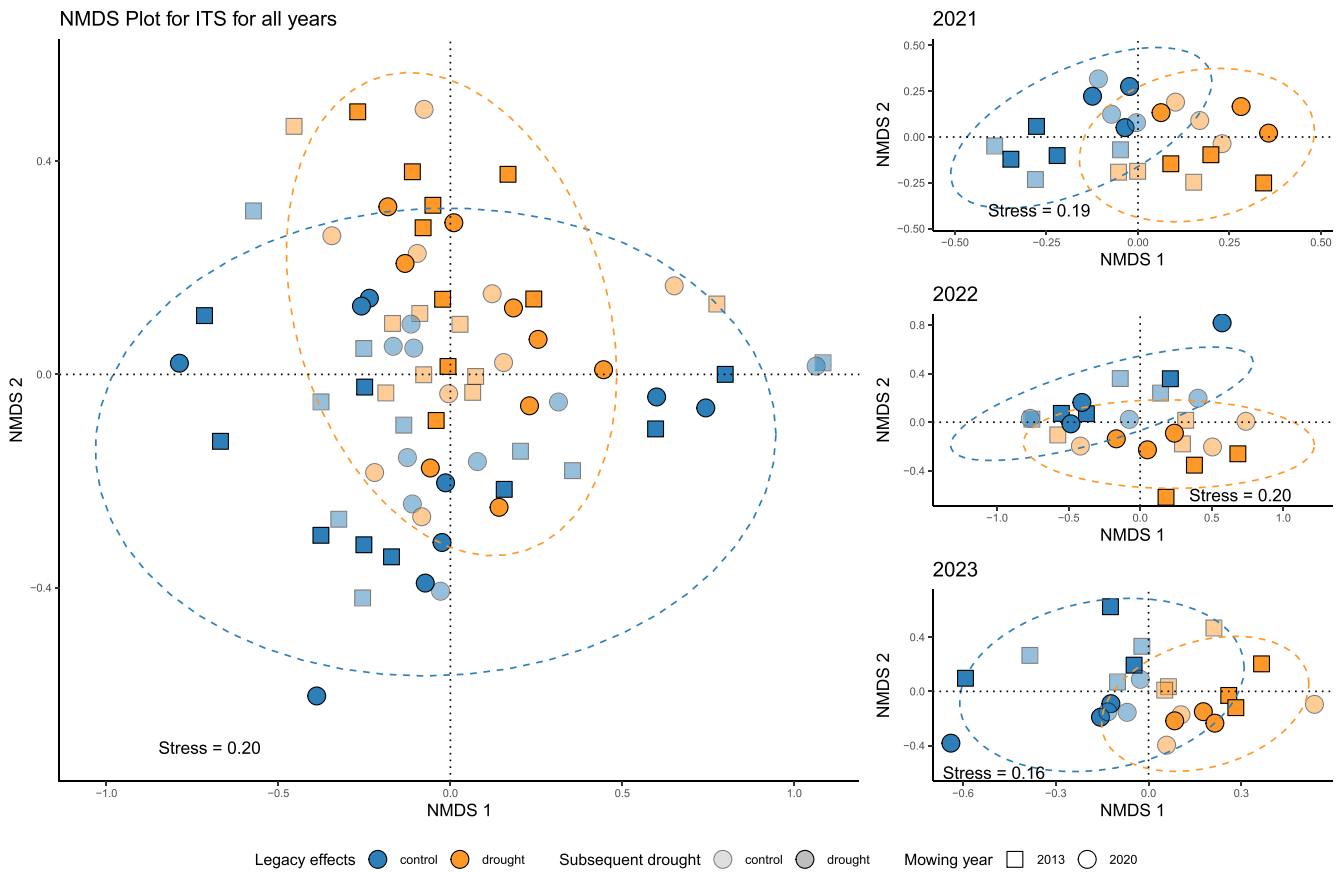


FIGURE 2 | Nonmetric dimensional scaling of soil fungal communities based on ITS sequencing and Bray–Curtis distances in response to drought legacy, subsequent drought and mowing year for all three sampled years, as well as separated diagrams for each sampled year (2021, 2022 and 2023). On the left are represented three sampled years together ($n = 72$) and on the right individual NMDS for each sampling campaign ($n = 24$). Each dot represents one observation of the soil fungal community.

amount of moisture reduction in response to the subsequent drought was dependent on the legacy effect of drought and on mowing year (Table S1, Subsequent drought*Legacy*Mowing year $\chi^2 = 19.18$ and $p < 0.001$). The biggest reduction in soil moisture was found in plots with drought legacy that were mowed in 2013, and the smallest effect of subsequent drought on soil moisture was found in the recently mowed plots of 2020 (Figure S3). There was also a significant three-way interaction of drought legacy, mowing year and subsequent drought treatment on soil temperature during the growing season (Table S2, SD*L*MY $\chi^2 = 7.39$ and $p = 0.006$). Post hoc tests showed that the temperature at soil level (0 cm) was higher in the subsequent drought treatment in the 2013 mowed plots with drought legacy and in the 2020 mowed plots without drought legacy (Figure S5). Plant-available N and P in soils were higher in the 2020 mowed plots, and the subsequent drought treatment tended to increase plant-available P, while having no effect on plant-available N (Figure S7). Moreover, subsequent drought tended to increase DON and DOC, while only DOC was affected by mowing year, with the highest concentrations found for the recently mowed plots of 2020. Drought legacy significantly decreased microbial N but only had a marginally significant effect on microbial C, and both microbial N and C pools were not affected by the subsequent drought treatment or mowing year (Table S3).

3.2 | Soil Microbial Communities Respond Stronger to Drought Legacy Than to Subsequent Drought Treatment

Soil fungal community composition responded strongly to drought legacy in interaction with mowing year (PERMANOVA L*MY $F_{1,71} = 1.67$, $p = 0.009$, Table S4), but was not affected by subsequent drought (SD $F_{1,71} = 1.08$, $p = 0.213$, Table S4). There were two marginally significant interactions for factors determining fungal community composition: between mowing year and sampled year (MY*Y $F_{2,71} = 1.25$ $p = 0.06$, Table S4) and between drought legacy and subsequent drought (L*SD $F_{1,71} = 1.40$ $p = 0.06$, Table S4), but interestingly there was no significant three or four-way interaction between all predictive variables (Table S4). When the soil fungal communities for each year were analysed separately, the ordinations show a clear separation of these communities by drought legacy (colours blue and orange in Figure 2 refer to drought legacy and control), but when all samples are shown together, this pattern becomes less clear (Figure 2). Furthermore, we found that the amount of variation in fungal community composition explained by mowing year increased from 2021 to 2023, while the variation explained by the subsequent drought treatment decreased slightly, as well as the variation explained by drought legacy (R^2 values in Figure S8).

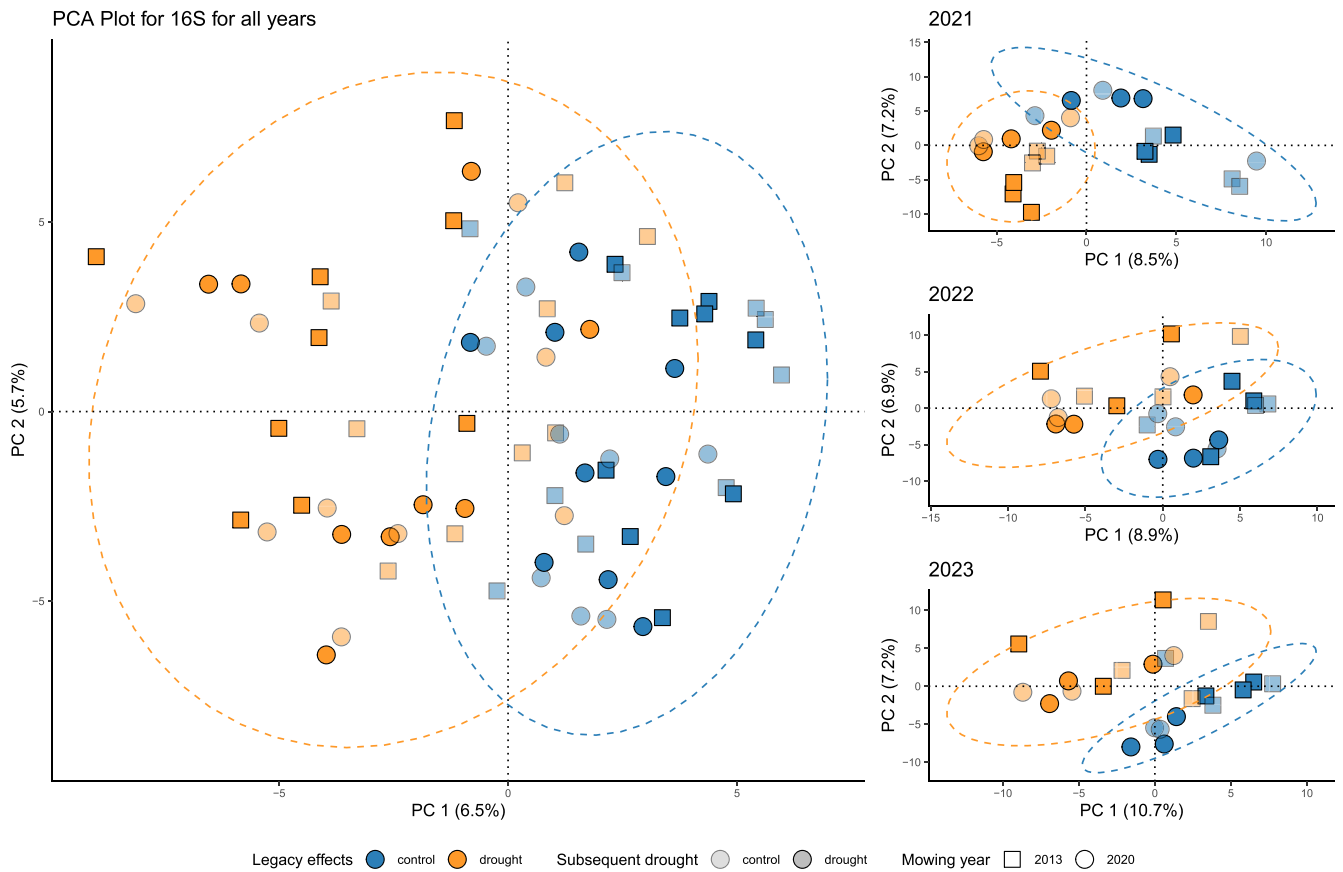


FIGURE 3 | Ordination diagrams (PCA) for soil bacterial communities based on 16S amplicon sequencing and clr-transformed data in response to drought legacy, subsequent drought and mowing year for all three sampled years as well as separated diagrams for each sampled year (2021, 2022 and 2023). On the left are represented the three sampled years together ($n = 72$) and on the right, individual diagrams for each sampling campaign ($n = 24$). Each dot represents one observation of the soil bacterial community.

Soil bacterial community composition was strongly shifted by drought legacy, in interaction with mowing year and the subsequent drought treatment (Table S5 PERMANOVA L^*MY*SD $F_{1,71} = 1.11$ $p = 0.05$). There was no significant four-way interaction of these three variables with the sampled years (Table S5 PERMANOVA $L^*SD*MY*Y$ $F_{2,71} = 0.90$ $p = 0.70$); however, sampled years significantly interacted separately with mowing years and drought legacy to determine soil bacterial community composition (Table S5). When soil bacterial communities for each sampled year were analysed separately, the ordination diagrams show a clear separation of communities by drought legacy (Figure 3). These effects did not change with sampling year, with most variation in bacterial communities being consistently explained by drought legacy, followed by mowing year and the subsequent drought treatment (R^2 values in Figure S8).

3.3 | Drought Legacy, Subsequent Drought and Mowing Affect Plant Community Composition

Plant community composition responded strongly to drought legacy, in interaction with subsequent drought and mowing year across the three sampled years (PERMANOVA L^*SD*MY $F_{1,71} = 2.38$, $p = 0.033$, Table S6). There was no significant effect of a four-way interaction with sampled year on plant community composition ($L^*SD*MY*SY$ $F_{2,71} = 0.12$, $p = 0.99$, Table S6).

In general, the 2013 mowed control plots were dominated by *Calluna*, and the 2020 mowed plots showed a higher diversity of plant species, with *Molinia caerulea* and *Rumex acetosella* becoming more dominant in plots with drought legacy (Figure 4).

When analysed separately, *Calluna* cover responded strongly to the subsequent drought treatment, and this response was dependent on when the plots were mowed, but not on the legacy effect of drought (Table 1). In the 2013 mowed plots, *Calluna* cover was generally lower under subsequent drought, independent of the legacy effect of the long-term drought (Figure S9). For the plots mowed in 2020, *Calluna* cover was higher under subsequent drought in sampling years 2021 and 2022, but not different from the control in the final sampling year of 2023, and not affected by drought legacy (Figure S9).

3.4 | CO₂ Flux Response to Subsequent Drought Depends on Drought Legacy and on Mowing Years

The response of NEE to subsequent drought depended on the legacy of drought and on mowing year, with a significant three-way interaction (Table 2, Figure 5). For the 2013 mowed plots, the subsequent drought treatment led to less ecosystem C uptake (i.e., less negative NEE) compared to control ($p < 0.01$), and this was irrespective of the legacy effect of drought ($p = 0.99$). There

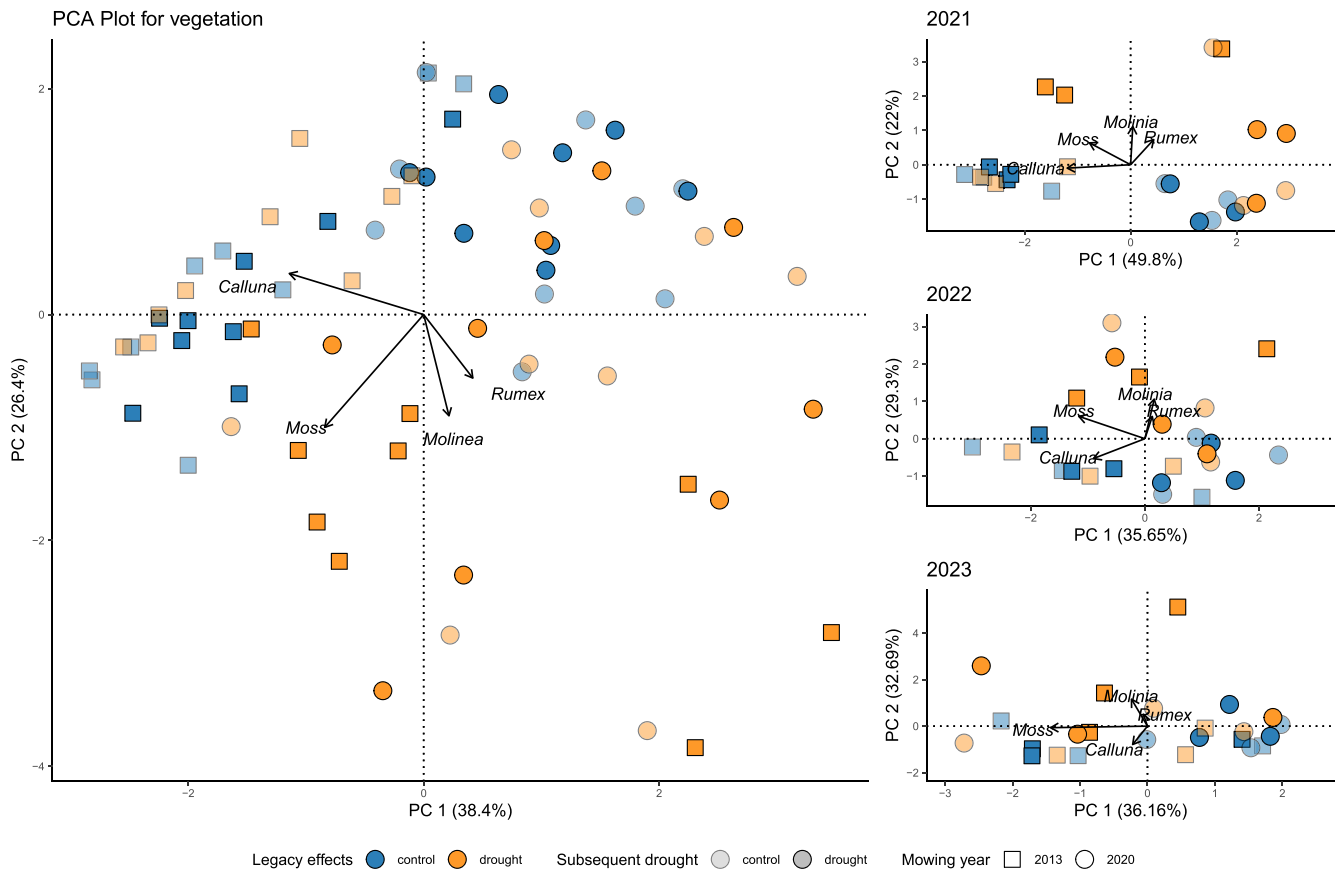


FIGURE 4 | Ordination diagram for the plant community composition in response to drought legacy, subsequent drought and mowing years for all three sampled years as well as separated diagrams for each sampled year. Plant cover data were log-transformed prior to ordination analysis; the arrows on each diagram display the four most influential variables for each ordination: Moss represents all bryophytes, Calluna stands for *Calluna vulgaris*, Molinia for *Molinia caerulea* and Rumex for *Rumex acetosella*.

TABLE 1 | ANOVA table of *Calluna*'s cover in response to drought legacy (L), subsequent drought (SD) and mowing year (MY) for the three sampled years (2021, 2022 and 2023) Statistically significant factors are highlighted in bold.

	χ^2	Df	<i>p</i>
Legacy	0.301	1	0.583
Subsequent drought	9.012	1	0.003
Mowing year	141.545	1	< 0.001
SD*L	0.915	1	0.339
L*MY	0.878	1	0.349
SD*MY	12.611	1	< 0.001
L*SD*MY	0.299	1	0.584

was, on average, less C uptake in the 2020 plots than in the 2013 plots (Figure 5). For the 2020 plots, post hoc tests showed no difference in NEE between subsequent drought and control, or between drought legacy and no drought legacy.

The response of ER to subsequent drought depended on drought legacy and on mowing year, but there were no significant three-way interactions (Table 2, Figure 5). ER was reduced by subsequent drought in plots mowed in 2013 (post hoc $p=0.001$),

irrespective of drought legacy (post hoc $p=0.87$), while in 2020, in mowed plots, there was no effect of drought legacy or subsequent drought (Figure 5). In plots mowed in 2020, ER did not differ between drought legacy and control (post hoc $p=0.07$) or between subsequent drought and control (post hoc $p=0.23$). The response of photosynthesis to subsequent drought was not affected by drought legacy, but it was by mowing year (Table 2). Subsequent drought only reduced photosynthesis in the 2013 mowed plots, but these rates were still higher than in the 2020 plots (Figure 5). Similarly, overall, plots mowed in 2013 had higher photosynthesis than the 2020 mowed plots in both drought legacy and control (Figure 5).

3.5 | Drought Legacy Directly Affects Ecosystem Respiration but Not NEE CO₂ Fluxes

We tested our hypothesised relationships between drought treatments, plant and soil microbial communities and ecosystem CO₂ fluxes in two separate SEMs. The SEM for mechanisms governing NEE showed that drought legacy did not explain NEE CO₂ fluxes; but that these fluxes were solely driven by plant community composition (Figure 6a, Fischer's $C=24.04$, $p=0.45$ and $df=24$). Plant community composition, that is, PCA axis 1 scores, was positively related to NEE, indicating that a higher *Calluna* cover leads to a more negative NEE (more ecosystem C uptake). The fixed effects in our SEM explained 42%

TABLE 2 | ANOVA results from linear mixed-effect models testing for the fixed effects of drought legacy (L), subsequent drought (SD) and mowing year (MY) on the repetitive measures of CO₂ fluxes from ecosystem respiration, net ecosystem exchange and photosynthesis across three growing seasons (2021, 2022 and 2023). Statistically significant factors are highlighted in bold.

	Chisq	Df	p
Ecosystem respiration			
Legacy	1.265	1	0.261
Subsequent drought	2.508	1	0.113
Mowing year	250.443	1	< 0.001
L*SD	6.330	1	0.012
L*MY	15.422	1	< 0.001
SD*MY	10.295	1	0.001
L*SD*MY	1.922	1	0.166
Photosynthesis			
Legacy	0.000	1	0.994
Subsequent drought	19.920	1	< 0.001
Mowing year	286.971	1	< 0.001
PAR	10.249	1	0.001
L*SD	0.039	1	0.843
L*MY	6.230	1	0.013
SD*MY	24.526	1	< 0.001
L*SD*MY	3.279	1	0.070
Net ecosystem exchange			
Legacy	1.102	1	0.294
Subsequent drought	22.381	1	< 0.001
Mowing year	230.281	1	< 0.001
PAR	14.320	1	< 0.001
L*SD	2.083	1	0.149
L*MY	3.862	1	0.049
SD*MY	23.627	1	< 0.001
L*SD*MY	4.671	1	0.031

of variation in NEE, while accounting for the yearly and plot random variation increased this to 58%. Our model further showed that although soil bacterial communities were strongly and directly affected by drought legacy, by plant community composition, and by changes in temperature, they did not predict NEE (Figure 6a). Plant community composition was further positively related to temperature; that is, temperature just above the soil surface was higher in plots with lower *Calluna* cover, and this also affected bacterial communities but had no effect on NEE.

The second SEM showed that drought legacy increased ecosystem respiration directly (Fischer's C = 14.21, $p = 0.71$ and

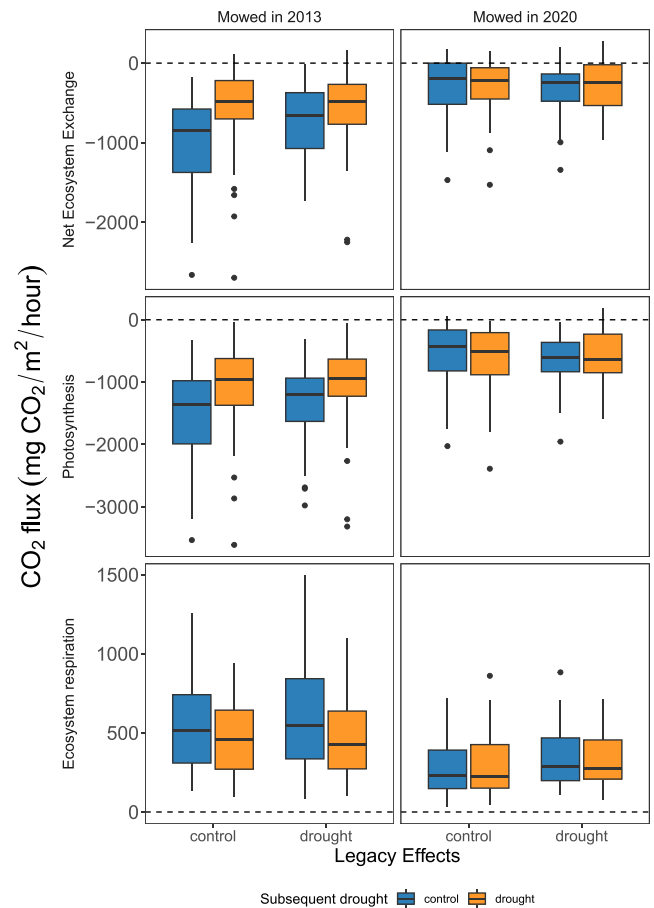


FIGURE 5 | CO₂ fluxes measured in the long-term drought experiment in response to subsequent drought and control, across three growing seasons (2021, 2022 and 2023). On the x-axis are the long-term legacy effects of drought and the colours represent the experimental conditions of the subsequent drought (orange for drought and blue for control). CO₂ flux values for ecosystem respiration come from direct measurements on dark chambers and net ecosystem exchange (NEE) comes from transparent chamber measurements, while photosynthesis values have been calculated as the difference between NEE and ER (photosynthesis = NEE - ER).

df = 18, Figure 6b). The fixed effects of this model explained 58% of the variation in ER, while accounting for the yearly and plot random variation increased this to 77%. In this model, plant community composition was negatively related to ER, meaning that plots with a higher *Calluna* cover also had higher ER (Figure 6b). There were no significant pathways linking soil bacterial or fungal communities to ER, even though soil bacterial communities were directly affected by plant communities and drought legacy.

4 | Discussion

We set out to test how chronic drought affects ecosystem response to a subsequent drought, focussing on its ability to take up CO₂, using a unique, long-term drought experiment in a heathland ecosystem. Specifically, we wanted to test how the age of the vegetation determines ecosystem response to drought and the consequences for ER and NEE. We hypothesised that

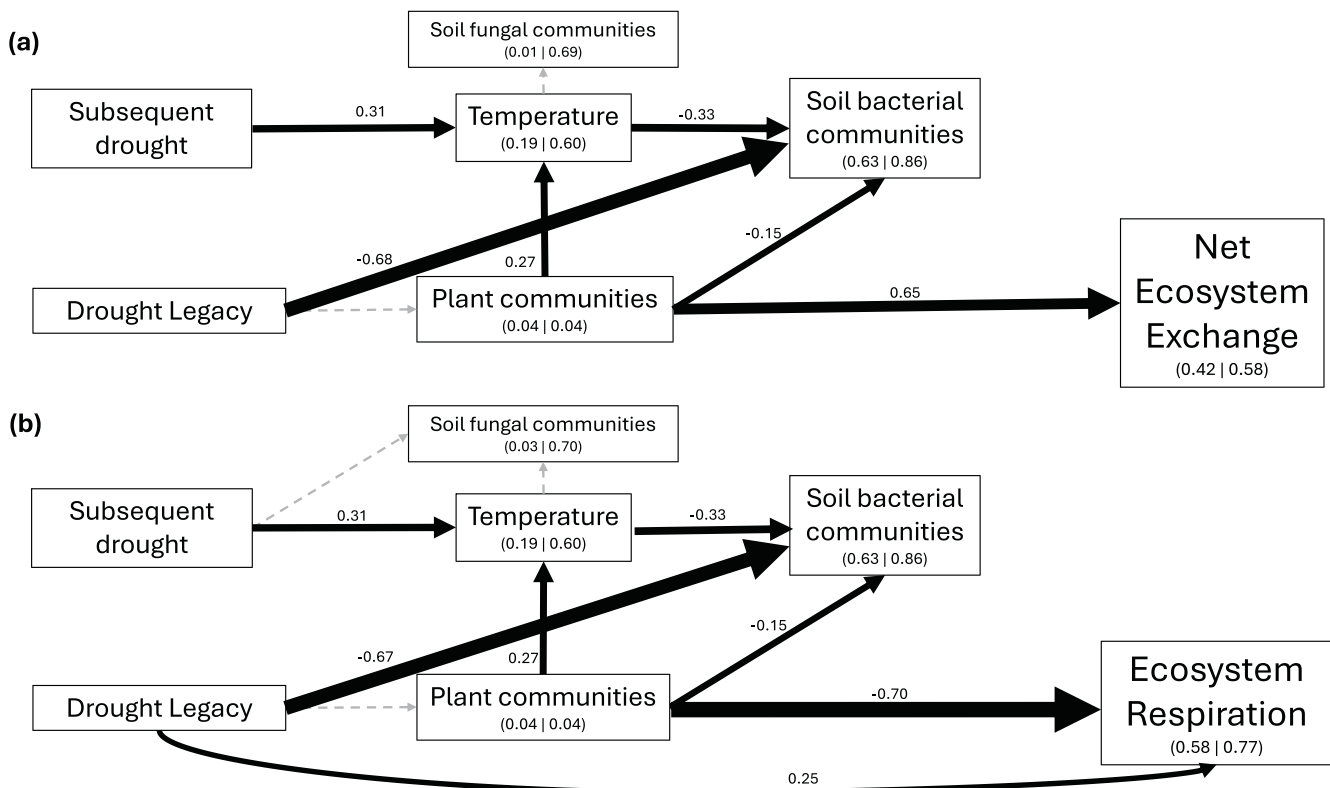


FIGURE 6 | Structural equation models depicting the direct and indirect effects of drought legacy, subsequent drought, temperature, plant and soil microbial communities on CO₂ fluxes from net ecosystem exchange (a) and from ecosystem respiration (b). Plant communities are represented by the scores of the first axis of a PCA ordination based on plant cover, soil bacterial communities are represented by sample scores of the first axis of a PCA ordination and soil fungal communities are represented by sample scores on the first NMDS axis. Values inside each box, represented by brackets underneath variables, represent the total variance explained by fixed effects only and by the combination of fixed and random (block and year) effects in the models. Black filled arrows represent significant paths, while grey dashed arrows represent nonsignificant paths that were retained on the final model. Values above arrows represent path coefficients, and the width of arrows is proportional to their strength.

previous chronic drought (drought legacy) would reduce the impact of a subsequent drought on ER through shifts in microbial community composition, and that drought legacy would increase the impact of a subsequent drought on both ER and NEE most strongly in older heathland vegetation. We found that chronic drought persistently shifted soil bacterial and fungal communities, but that the subsequent drought only had a minor impact on these communities. Ecosystem respiration was reduced by subsequent drought, but this did not depend on drought legacy. In line with our hypothesis, the cover of the dominant *Calluna* vegetation was reduced strongly by subsequent drought in older *Calluna* but not in young *Calluna* (1 year old, mown in 2020). Similarly, photosynthesis and NEE were reduced under subsequent drought in older *Calluna*, and this effect did not depend on previous chronic drought. These findings show a mismatch between aboveground and belowground responses to drought. Despite strong and persistent shifts in soil microbial communities as a result of 20 years of summer drought, ecosystem C fluxes under a subsequent drought were determined by vegetation responses. Our findings also show that older heathlands are more strongly affected by drought, reducing their capacity to take up CO₂ in the crucial phase during which ecosystem C stocks are accumulated.

Chronic drought caused strong and persistent shifts in soil bacterial and fungal communities, which is consistent with

previous findings (Canarini et al. 2021; Gliesch et al. 2024). For both bacterial and fungal communities, the effect of chronic drought depended on mowing year, confirming that indirect effects via plant responses are also driving soil microbial community response to drought (de Vries et al. 2018; Fahey et al. 2020). Moreover, we only found an effect of subsequent drought on bacterial communities, and this depended on drought legacy and mowing year. No effect of subsequent drought was found on fungal communities, which is consistent with those being better able to cope with drought than bacteria (Barnard et al. 2013; de Vries et al. 2018). The complete absence of the effect of subsequent drought on fungal communities during our sampling years—also in those that were not exposed to previous chronic drought—suggests that the impact of drought on these communities in heathlands takes more than 3 years to establish, likely because these systems are relatively dry (Knight et al. 2024). However, while bacterial communities were more responsive to subsequent drought, variation in fungal communities explained by mowing year and drought legacy was higher than for bacteria. This suggests that while fungal communities are better able to resist change, once they are altered, they do not return to their original state (Cordero et al. 2023).

Similar to belowground communities, drought legacy caused persistent shifts in plant communities. In contrast to our hypothesis, drought legacy—in particular in combination with

continued drought and in the plots mown in 2020—induced a shift towards increased abundance of the fast-growing species *Molinia caerulea* and *Rumex acetosella*. In the recently mown plots, this may be a consequence of aboveground and belowground competitive release through the elimination of *Calluna* cover and higher nutrient availability (Figure S8). Fast-growing species have been shown to be able to regrow quickly after drought and sometimes gain dominance through their competitive traits that enable them to capitalise on the increased nutrient availability after soil rewetting (de Vries et al. 2018; Hoover et al. 2014; Liu et al. 2018). However, at the same time and in contrast with our initial hypothesis, *Calluna* cover was reduced by subsequent drought only in the building stage (plots mown in 2013), and not by drought legacy (Figure S9). These findings indicate that building-stage *Calluna* is vulnerable to drought initially, but that this effect may level off when drought persists, which was visible in the third year of our sampling (Figure S9). These findings partly support our suggested mechanism of greater vulnerability to drought in older plants but also suggest that *Calluna* plants may adapt to chronic drought, potentially through deeper rooting, which we did not measure here. They also suggest that this reduction in *Calluna* cover, even if only temporary, might facilitate an increase in fast-growing species under drought and may result in a lower build-up of soil organic matter (Gliesch et al. 2024).

In line with the effects of drought on *Calluna* cover, we found that subsequent drought reduced NEE in building stage *Calluna* (plots mown in 2013), and that this effect did not depend on drought legacy. Similarly, ecosystem respiration was reduced by subsequent drought only in plots mown in 2013, but drought legacy had no effect. These findings indicate a mismatch between plant and soil community responses to chronic drought and ecosystem C cycling. The strong shifts in soil communities in response to exposure to 20 years of summer drought but the absence of a relationship with ecosystem respiration, suggest that shifts in soil communities might be an adaptation to drought that maintains their functioning. For example, a single drought has been shown to shift soil microbial communities towards fungal dominance, increasing the resistance of soil functioning to a second drought (de Vries et al. 2012). In addition, while 1 year of drought altered the activity of several enzymes involved in the C cycle, 10 years of drought shifted them back to control levels, while bacterial and fungal community composition continued to diverge from the control (Canarini et al. 2021). In contrast to the lack of relationships between soil bacterial and fungal communities and ecosystem C fluxes, plant community composition (represented by PC1 scores of the PCA of plant community composition in the SEM, Figure 6) was the primary driver of both NEE and ecosystem respiration. PC-axis 1 was mostly associated with shifts in *Calluna* cover (Figure 5), which was reduced with subsequent drought in the plots mown in 2013, but was not affected by drought legacy. NEE became more positive with increasing PC1 scores (indicating less ecosystem CO₂ uptake), along which *Calluna* cover decreased, while ecosystem respiration decreased along this axis too. These results show that *Calluna* cover drives ecosystem C fluxes in this system, overruling any effect of changes in soil bacterial and fungal communities. This is in line with previous results from the same field experiment that show that long-term summer drought resulted in a decrease in soil C underneath old *Calluna* (Gliesch

et al. 2024), and that microbial communities had adapted to this long-term drought by regaining their activities rapidly after rewetting but respiring relatively less C (de Nijs et al. 2019). However, while we measured C fluxes repeatedly over three growing seasons, we only had one observation for plant and microbial communities per year, which might obscure any relationships between communities and C fluxes on shorter time scales.

Taken together, the results from this unique, long-term (> 20 years) field experiment show that chronic summer drought strongly changes plant and soil microbial communities, but that there is a mismatch between these changes and ecosystem C fluxes. The strongly altered soil bacterial and fungal communities in response to chronic drought showed no sign of recovery 3 years after ending the chronic drought, nor did they affect ecosystem C fluxes under a subsequent drought. In contrast, shifts in plant community composition best predicted ecosystem C fluxes, with older, building-stage *Calluna* (mown in 2013) being most vulnerable to the effects of a subsequent drought. While our results are limited to one single site, these findings give insight into ecosystem response to long-term drought and the consequences for C cycling; knowledge that is pivotal for predicting climate change feedbacks under a changing climate.

Author Contributions

Mariana Gliesch: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing – original draft, writing – review and editing. **Leonardo Hinojosa Sanchez:** data curation, investigation, methodology, writing – review and editing. **Kiki Boreel:** data curation, formal analysis, writing – review and editing. **Albert Tietema:** conceptualization, methodology, resources, supervision, writing – review and editing. **Franciska T. de Vries:** conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing – original draft, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study is openly available via Figshare on <https://doi.org/10.21942/uva.28287131>. All the raw DNA sequences from this project have been deposited at NCBI database for SRA under project number PRJNA1216416.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Picture of an actual experimental plot in the study site in Oldebroek, the Netherlands. This experiment featured in episode 5 of 'Govert naar de kern van de aarde', a popular scientific series on the functioning of the Earth system on Dutch national television, 8 October 2023 (https://www.npostart.nl/govert-naar-de-kern-van-de-aarde/08-10-2023/VPWON_1344387). **Figure S2:** A priori structural equation model for (A) Net Ecosystem Exchange (NEE) and (B) Ecosystem Respiration (ER). **Table S1:** ANOVA table from mixed-effect models testing for the effects of drought legacy (L), subsequent drought (SD), mowing years (MY) and their interactions on soil volumetric water content during the growing season across three sampled years (2021, 2022 and 2023). **Figure S3:** Average ratio of soil

volumetric water content during the growing season for the three sampled years in the mowed parts of the plot of 2013 and 2020. **Figure S4:** Soil volumetric water content (ratio) recorded by TOMST sensors and averaged every 12h for the years of 2021, 2022 and 2023 for plots mowed in 2013 and 2020 that had a legacy of drought or that were kept as control, colour coded by the subsequent drought treatment (blue for control and orange for drought) with a $n = 3$. **Figure S5:** Estimated marginal means of temperature at soil level (0cm) during the growing season for the three sampled years in the mowed parts of the plot of 2013 and 2020 in response to the subsequent drought treatment and the legacy effects of drought. **Figure S6:** Temperature at soil level (0cm) recorded by TOMST sensors and averaged every 12h for the years of 2021, 2022 and 2023 for plots mowed in 2013 and 2020 that had a legacy of drought or that were kept as control, colour coded by the subsequent drought treatment (blue for control and orange for drought) with a $n = 3$. **Table S2:** ANOVA table from mixed-effect models testing for the fixed effects of drought legacy (L), subsequent drought (SD), mowing years (MY) and their interactions on temperature at soil level (0cm) during growing season across 3 years (2021, 2022 and 2023). **Figure S7:** Plant-available nutrients in response to drought legacy, subsequent drought and mowing year across three sampled years. ANOVA results indicate that only the subsequent drought and mowing year are significant. **Table S3:** ANOVA table from linear mixed-effect models testing for the fixed effects of drought legacy, mowing years, subsequent drought and sampled year on different soil parameters. Statistically significant effects (where $p < 0.05$) are highlighted in bold. **Figure S8:** Variation explained by the different variables (Mowing Years, Subsequent drought and Drought Legacy) in the PERMANOVA for soil bacterial (based on 16S sequencing, with clr-transformed dataset and Euclidean distances) and fungal (based on ITS sequencing with Bray–Curtis distances) communities analysed for each sampled year (2021, 2022 and 2023) separately. **Table S4:** PERMANOVA table for the effects of drought legacy (L), subsequent drought (SD), mowing years (MY) and sampled year (Y) on the composition of soil fungal communities based on ITS sequencing data. Statistically significant factors and values are highlighted in bold. **Table S5:** PERMANOVA table for the effects of drought legacy (L), subsequent drought (SD), mowing years (MY) and sampled year (Y) on the composition of soil bacterial communities based on 16S sequencing data. Statistically significant factors and values are highlighted in bold. **Table S6:** PERMANOVA table for the effects of drought legacy (L), subsequent drought (SD), mowing years (MY) and sampled year (Y) on the composition of plant communities based on log-transformed percentage cover data. Statistically significant factors and values are highlighted in bold. **Figure S9:** Calluna's cover at peak biomass in response to subsequent drought, drought legacy and mowing years across the three sampled years (2021, 2022 and 2023). **Figure S10:** Measurements from 12 August 2021. **Figure S11:** Measurements from 27 August 2021. **Figure S12:** Measurements from 1 October 2021. **Figure S13:** Measurements from 18 October 2021. **Figure S14:** Measurements from 29 March 2022. **Figure S15:** Measurements from 26 April 2022. **Figure S16:** Measurements from 31 May 2022. **Figure S17:** Measurements from 30 June 2022. **Figure S18:** Measurements from 29 July 2022. **Figure S19:** Measurements from 24 August 2022. **Figure S20:** Measurements from 4 October 2022. **Figure S21:** Measurements from 26 October 2022. **Figure S22:** Measurements from 3 May 2023. **Figure S23:** Measurements from 6 June 2023. **Figure S24:** Measurements from 12 July 2023. **Figure S25:** Measurements from 9 August 2023. **Figure S26:** Measurements from 13 September 2023.